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Palmer, I., Clarke, S. A., Nelson, J., Schatton, W., Dunne, N. J., & Buchanan, F. (2012). Identification of a suitable sterilisation method for collagen derived from a marine Demosponge. *International Journal of Nano and Biomaterials*, 4(2), 148-163. <https://doi.org/10.1504/IJNBM.2012.050306>

### **Published in:**

International Journal of Nano and Biomaterials

### **Document Version:**

Peer reviewed version

### **Queen's University Belfast - Research Portal:**

[Link to publication record in Queen's University Belfast Research Portal](#)

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## IDENTIFICATION OF A SUITABLE STERILISATION METHOD FOR COLLAGEN DERIVED FROM A MARINE DEMOSPONGE

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**DR. SUSAN CLARKE** has been a biomedical scientist involved in orthopaedic research for more than ten years, particularly the biological response to synthetic bone substitutes. Dr Clarke is an experienced histologist and cell biologist with interest in human and animal *in vitro* culture methods, including the isolation and expansion of bone marrow stromal cells. Currently her research interests are focussed on biomimetics and marine biotechnology; taking inspiration from marine organisms as

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novel scaffolds for bone tissue replacement or *ex vivo* bone tissue engineering.

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**DR. WOLFGANG SCHATTON** earned his doctoral degree in Pharmacology and Medicinal Chemistry from the University of Frankfurt, Germany. He has held several academic appointments as an adjunct professor, has led numerous research projects and authored over 30 scholarly articles and patents. His career in the pharmaceutical industry includes over 15 years as head of R&D at Merz Pharmaceuticals and Pascoe Naturmedizin and a successful track record for developing and bringing drugs to market. He has been responsible for numerous pharmaceutical product approvals and new product launches in the fields of tumour angiogenesis and infectious, cardiovascular, and neurodegenerative disease. In 1993 he co-founded KliniPharm to develop technologically innovative natural products based on the latest scientific research. His ties to industry and academia have led to the formation of collaborations with corporate partners and scientific institutions throughout the European Union, from which KliniPharm's patents, product prototypes, and product developments have emerged.

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**PROFESSOR FRASER BUCHANAN** has been involved in various aspects of materials degradation research for the past 20 years. His main area of research is biomaterials such as calcium phosphate ceramics and

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bioresorbable polymers used in bone repair applications. His current research on calcium phosphates focuses on injectable calcium phosphate cements and development of naturally derived bioresorbable calcium phosphates. His research on bioresorbable polymers focuses on synthetic biocompatible polymers (e.g. aliphatic polyesters) such as polylactic acid (PLA) and polyglycolic acid (PGA) and techniques to monitor and control their bioresorption profile.

**ABSTRACT:** Collagen is widely used as a biomedical material, and its importance is likely to grow as research and understanding progresses in this field. As a biomedical material, ensuring the sterility of collagen before use as, or incorporation into, a medical device is paramount. However, common sterilisation techniques can induce changes in the physical structure and protein chemistry of collagen, potentially affecting the performance. In this preliminary study the influence of autoclaving, gamma irradiation and ethylene oxide gas sterilisation on the denaturation temperature and helical content of the collagen was evaluated using differential scanning calorimetry and Fourier transform infrared spectroscopy. Early results indicate that all sterilisation techniques affect collagen properties but suggest that the least damaging of the techniques investigated was  $\gamma$  irradiation.

**KEY WORDS:** Sterilisation; Collagen; *Chondrosia reniformis*; Autoclave; Gamma Irradiation; Ethylene Oxide; Denaturation; Fourier Transform Infrared Spectroscopy; FTIR; Differential Scanning Calorimetry; DSC; Chain Scission; Cross Linking; Helical Content;

## INTRODUCTION

Collagens are a family of fibrous proteins found in all multi-cellular animals and are amongst the most abundant proteins in the animal kingdom (Alberts *et al.*, 2002); (Campbell and Reece, 2002). As a major component of skin and bone in humans, collagens make up approximately 25 % of our total protein mass (Alberts *et al.*, 2002). Molecules of collagen consist of three polypeptide chains, known as  $\alpha$ -chains, which are wound around one another to form a triple helix (Alberts *et al.*, 2002).

This prevalence of collagen in human tissues makes it an ideal candidate as a polymer for tissue engineering matrices and other biomedical materials. The use of collagen as a biomaterial was reported as early as the 1970s (Chvapil, 1977) and since then, research has been carried out on its use as a graft for the treatment of burns (Burke *et al.*, 1981) and other skin wounds (Eaglstain and Falanga, 1997), as a scaffold to facilitate the healing of ligament (Meaney Murray *et al.*, 2003) and bone (Pek *et al.*, 2008) and as an additive for bone substitutes (Zerwekh *et al.*, 1992). Properties of collagen, such as minimal antigenicity, excellent biocompatibility (Song *et al.*, 2006), the potential for drug delivery (Friess, 1998) and effects on cellular responses such as the acceleration of osteoconduction (Sugaya *et al.*, 1990), suggest that its use as a biomedical material will become commonplace.

At present, the major sources of industrial collagen are bovine and porcine. These, however, carry the potential risk of transmissible spongiform encephalopathies and pathogens such as foot-and-mouth disease (Zhang, Liu, and Li, 2009). There is now a consensus that a safer alternative would be to use collagen from aquatic and, specifically, marine sources (Song *et al.*, 2006); (Giraud-Guille *et al.*, 2000). The marine Demosponge *Chondrosia reniformis* (Demospongiae: Chondrillidae: Chondrosiidae) (Nardo, 1847) is a common species that has been identified worldwide, has a high collagen content (Nickel and Brümmer, 2003) and a low risk of detrimental toxic compounds (Swatschek *et al.*, 2002). The fact that *C. reniformis* also reproduces asexually suggests that harvesting of the sponge for collagen isolation could be carried out on a commercial scale (Bavestrello *et al.*, 1998). Swatschek *et al.*, (2002) developed a method for isolating collagen from *C. reniformis* and demonstrated its suitability as a substitute for conventional collagen.

Prior to incorporation into, or use as, a biomaterial the sterility of the collagen must be ensured. Sterilisation aims to kill or inactivate any form of microbial contaminant. However, many common sterilisation techniques can result in changes in the physical properties, such as denaturation temperature, and chemical properties of collagen, such as the helicity. Changes to these properties may have potential effects on collagen's performance as a biomaterial.

Steam sterilisation, usually in the form of autoclaving, although not commonly used on an industrial scale, is the most widely used health care sterilisation technique due to its speed, efficacy and low cost (Wiegand *et al.*, 2009). The technique utilises pressurised steam and superheated water, thus providing moisture and heat, which are the two requisites for the thermal destruction of microbial life. However, it is

known that high temperatures can adversely affect sensitive biomolecules such as collagen. Placing a value on the denaturation temperature of collagen is highly dependent on the species. Vertebrates, for example, have at least 27 different types of collagen (Myllyharju and Kivirikko, 2004) with denaturation temperatures ranging from the approximately 30 °C up to 160 °C depending on species and location within the body (Zhang, Liu, and Li, 2009); (Wang *et al.*, 1999). *C. reniformis* belongs to a far less well studied phylum (Porifera) leading to a dearth of information on its collagen.

An alternative to autoclaving is the use of gamma ( $\gamma$ ) irradiation. It is the most common sterilisation technique used in the orthopaedic implant industry (Collier *et al.*, 1996), where doses of between 25 and 40 kGy guarantee sterility. Energy from photons of  $\gamma$  radiation is transferred to electrons in the target material. This yields highly active electrons and thus highly reactive free radicals which induce breaks in the DNA double helix of any microorganisms present. This prevents replication and therefore leads to sterility. However, it has been shown that  $\gamma$  irradiation can cause both crosslinking (Jabbari and Nozari, 2000) and chain scission (Goldman *et al.*, 1996) in polymers including collagen (Olde Damink *et al.*, 1995; Wiegand *et al.*, 2009).

The final sterilisation method considered in this study was the use of ethylene oxide gas (EtO). It is an alkylating agent, which causes powerful alkylation reactions with cellular constituents, such as nucleic acids and functional proteins such as enzymes, of any microorganisms present, resulting in denaturation (Mendes, Brandão, and Silva, 2007). Using EtO is considered by some as the only acceptable sterilisation method for sensitive medical devices and has been described as the “most cost-effective, low temperature sterilisation process available” (Mendes, Brandão, and Silva 2007, p.574). It does however have some limitations, such as its flammable and explosive nature, its cost and the lengthy cycle time of the process (Rutala and Weber, 1999). A significant reason behind the lengthy cycle time is the need for adequate aeration or degassing in order to reduce the risk of residual gas which can compromise biocompatibility of the material (Vink and Pleijsier, 1986). In addition, EtO is known to react with amino acids present in collagen resulting in decreased helical stability (Olde Damink *et al.*, 1995) and it may cause alterations which reduce protein activity (Pekkarinen *et al.*, 2004).

In general, all sterilisation techniques have the potential to significantly affect both the biological activity and physical properties of biomolecules such as collagen. Therefore, the selection of a sterilisation technique should be made only following an investigation into the possible

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effects of the said technique on the material of interest. In this study, the influence of sterilisation of collagen from the marine Demosponge *C. reniformis* by autoclaving,  $\gamma$  irradiation and EtO gas treatment was investigated.

## **MATERIALS & METHODS**

### **Materials**

Collagen from *C. reniformis* was obtained in the form of compressed fibres from KliniPharm GmbH (Frankfurt, Germany) who have a strong research background in the collagen of this species. Extraction of collagen from *C. reniformis* was based on a method originally described by Diehl-Seifert *et al.* (1985) and later modified by Swatschek *et al.* (2002). The exact details of the extraction and isolation processes used by KliniPharm however, are not in the public domain.

Aliquots of approximately 30 g of as received marine collagen were prepared for sterilisation. All samples were packaged in Duo-Check<sup>®</sup> sterilisation pouches with both steam and EtO indicators (Crosstex<sup>®</sup>, New York, USA).

### **Sterilisation**

Autoclaving was carried out in accordance with ISO/TS 17665-2:2009 at 140 kN/m<sup>2</sup> steam pressure, yielding a temperature of 126°C, for 11 minutes using an Autoclave 2100 Extended benchtop model (Prestige Medical, Blackburn, UK). This standard provides guidance on the application of ISO 17665-1:2006, which specifies the requirements of a moist heat sterilization process for medical devices.

Sterilisation by  $\gamma$  irradiation was performed by Isotron (Applied Sterilisation Technologies, Synergy Health plc, Swindon, UK) at ambient temperature with Cobalt<sup>60</sup> as the source producing radiation dose of 29.5 kGy, in accordance with ISO 11137-1:2006 which states the requirements for development, validation and routine control of a sterilisation process for medical devices using radiation.

EtO gas sterilisation was also performed by Isotron in accordance with ISO 11135-1:2007, which states the requirements for development, validation and routine control of a sterilisation process for medical devices using EtO. The measurement of residual gas concentration was carried out in accordance with ISO 10993-7:2008, which specifies the allowable

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limits for residual EtO in sterilised medical devices and notes methods for determining compliance so that devices may be released. The process involves three phases. A pre-conditioning process is necessary which raises the temperature to within the range of 40 °C – 60 °C and the humidity to within the range of 45 % - 75 % for approximately 24 hours. Samples are then transferred to a sterilisation chamber which is evacuated and flushed with Nitrogen gas to remove air before the necessary moisture content is re-established within the chamber by steam injection. Finally EtO gas was introduced at the required concentration, maintained for a defined period, vented and then repeatedly flushed with Nitrogen or air to remove any remaining EtO from the chamber.

### **Differential Scanning Calorimetry (DSC)**

A DSC 6 differential scanning calorimeter (Perkin Elmer Inc., Massachusetts, USA) was used to determine the denaturation temperatures of the collagens investigated. Collagen samples were weighed accurately ( $\pm 0.1$  mg) into aluminium pans which were then closed by crimping before being scanned over the range of 30 °C – 120 °C at a heating rate of 5 °C/min with empty pans used as a reference. Thermal denaturation was considered as the endothermic transition and recorded as the peak temperature of maximum power absorption during denaturation which was indicated by a peak in the temperature heat flow curve. Three samples from each of the experimental conditions were scanned and the mean denaturation temperatures calculated from these scans.

### **Fourier Transform Infrared Spectroscopy (FTIR)**

All spectra were recorded from scans in the range of 4000  $\text{cm}^{-1}$  – 650  $\text{cm}^{-1}$  and a resolution of 4  $\text{cm}^{-1}$  using a Spectrum 100 FTIR spectrophotometer with ATR attachment (Perkin Elmer Inc., Massachusetts, USA). A total of 32 scans were carried out per spectrum and three samples were scanned for each experimental condition. The triple helix content of the collagen was estimated by calculating ratios of the depths of transmission troughs following a previously described method by Gordon *et al.* (1974). The bands considered were N–H stretching of amide A, C=O stretching for amide I and N–H deformations for amide II and III bands (Chang and Tanaka, 2002). Positioning, relative transmissions and wave numbers of these bands are shown in Figure 1. Peak absorbance levels for these regions were calculated using a baseline tangent from the maximum transmission value of the spectra at approximately 1800  $\text{cm}^{-1}$ . The trough



at  $1450\text{ cm}^{-1}$ , which corresponds to  $\text{CH}_2$  deformation, has been shown to remain unchanged following denaturation (Gordon *et al.*, 1974), therefore the ratio of this band to those of interest was used to normalise the results. These ratios were considered to be a measure of the helical content of the collagen and any reduction in these ratios from those of the as received collagen was considered a measure of denaturation.

### **Statistical Analysis**

All data are presented as means of three determinations with standard deviations recorded where appropriate. Statistical analyses were performed using one-way analysis of variance (ANOVA) following confirmation of the test's assumptions. Anderson-Darling tests were used to confirm that the residuals were normally distributed and Bartlett's tests were used to confirm homogeneity of the variances. Comparisons of means were carried out using a Dunnett's test. A probability value of  $\leq 0.05$  was considered significant. ANOVA and the associated tests to confirm its assumptions were performed using MINITAB® Student Release 14.11.1 (Minitab Inc., Coventry, UK). Dunnett's tests were performed using SPSS Statistics 17.0 (IBM Corporation, New York, USA).

## **RESULTS**

### **Differential Scanning Calorimetry**

Temperatures of the peak heat flows were extracted from thermal transition curves (Figure 2) for the treated collagens, and these were considered to reflect denaturation temperatures (Table 1). ANOVA and Dunnett's tests indicated that there was a treatment effect ( $p = 0.012$ ) and that autoclaving significantly affected the denaturation temperature (Table 1). Calorimetric enthalpy was also calculated from thermal transition curves (Figure 2) with ANOVA and Dunnett's tests again indicating that there was a treatment effect with both autoclaving ( $p = 0.025$ ) and EtO treatment ( $p = 0.006$ ) significantly affecting the enthalpy (Table 1).

### **Fourier Transform Infrared Spectroscopy**

FTIR spectra for the treated collagens were largely similar (Figure 3, note spectra have been displaced along the transmission axis for clarity). The extent of denaturation caused by each sterilisation technique can be shown

as a reduction in helicity, as determined by changes in the respective amide bands (Figure 4). Helicity values were calculated from ratios between troughs as described in the materials and methods section (Figure 5). These results show that, across all four amide bands,  $\gamma$  irradiated collagen displays the smallest deviation in helicity from that of the as received collagen. Also, across three of the four amide bands, EtO treated collagen displays the largest deviation from the helicity of the as received collagen with autoclaved collagen showing an intermediate reduction in helicity. ANOVA and Dunnett's statistical tests confirmed these observations (Table 2). When compared with the as received collagen, the  $\gamma$  irradiated collagen showed the least deviation in helicity with no significant difference between the helicity of the treated collagen and the helicity of the as received collagen in all four of the amide bands. Conversely, when EtO treated collagen was compared against as received collagen a significant difference was seen in three of the four amide bands. Autoclaved collagen was shown to be only marginally better in terms of percentage helicity than EtO treated collagen with a significant difference seen in two of the four amide bands.

## DISCUSSION

Sterilisation of medical devices and their components is essential before they can be used. However, common sterilisation techniques, such as autoclaving, exposure to  $\gamma$  irradiation, or EtO gas treatment, by design, can cause alterations to biomolecules. Due to the increasing use of active biomaterials such as collagen in medical devices, the effect of sterilisation techniques on such materials must be thoroughly investigated.

All of the sterilisation methods used in this study affected the structural properties of the collagen. Although the denaturation process of collagen is complex and not fully understood, its analysis requires an understanding of collagen on a molecular level (Brinkmann *et al.*, 2000). The triple helical structure of collagen is based on non-covalent interactions between vicinal polypeptide chains and arises from the abundance of the amino acids glycine, proline and hydroxyproline (Wiegand *et al.*, 2009). An  $\alpha$ -chain is composed of a series of triplet Gly-X-Y sequences where X and Y are commonly proline and hydroxyproline respectively, although they can be any amino acid (Alberts *et al.*, 2002). The three chains of the triple helix are held together by hydrogen bonds which link the peptide bond (NH) of a glycine residue

with a peptide carbonyl (C=O) group in an adjacent polypeptide. Covalent aldol crosslinks, which form between the C-terminus of one collagen molecule and the N-terminus of an adjacent molecule, stabilise the packing of collagen molecules side by side to form a fibril (Wiegand *et al.*, 2009).

Denaturation is a process during which proteins, such as collagen, become biologically inactive due to changes in their native conformation (Campbell and Reece, 2002). It's likely that hydrogen bonds connecting the three chains of the triple helix, cross-links interconnecting collagen molecules and peptide bonds forming the  $\alpha$ -helices all play important roles in the denaturation of collagen (Brinkmann *et al.*, 2000). Thermal denaturation is considered to be a relevant indication of collagen's potential future use as, or as part of, a biomaterial due to the decreased *in vivo* stability of partially denatured collagen (Yunoki, Suzuki, and Takai, 2003). For the purposes of this study, denaturation was considered as the point of maximum power absorption during DSC analysis and, for the FTIR analysis, any reduction in helicity from that of the as received collagen.

DSC analyses indicate that the denaturation temperature of collagen extracted from *C. reniformis*, before undergoing any sterilisation treatments, was  $\approx 86$  °C. During autoclaving, the collagen was exposed to a temperature of 121 °C, which is in excess of the collagen's denaturation temperature. The denaturation temperature of  $\approx 106$  °C, found for autoclaved collagen, which is significantly higher ( $p = 0.012$ ) than the denaturation temperature of as received collagen, is likely to be the result of structural changes in the collagen caused by the autoclaving process. Thermal denaturation of collagen initially results in the breaking of hydrogen bonds that stabilise the helical structure (Rochdi, Foucat, and Renou, 2000) and is thought to be followed by the breaking of inter- and intra-molecular crosslinks (Vangsness *et al.*, 1997). These processes result in the unfolding of the collagen helices (Vyazovkin, Vincent, and Sbirrazzuoli, 2007), but the mechanism by which these changes increase the denaturation temperature requires further investigation.

A decrease in denaturation temperature between the as received collagen ( $\approx 86$  °C) and the EtO gas treated collagen ( $\approx 73$  °C) was observed, but was not significant ( $p = 0.085$ ) (Table 1). This finding is however consistent with previous work where EtO treatment of collagen has been shown to lower the denaturation temperature of both ovine (Olde Damink *et al.*, 1995) and bovine (Friess, 1998) collagen. Such a decrease in denaturation temperature is a sign of decreased helix stability.

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Sterilisation using  $\gamma$  irradiation resulted in the smallest change to the denaturation temperature of the marine collagen, no significant difference was observed between the denaturation temperatures of the as received collagen and the  $\gamma$  irradiated collagen ( $p = 0.217$ ). This is in contrast to much of the work reported in the literature where radiation caused significant chain scission resulting in cleaved peptide bonds (Cheung *et al.*, 1990), produced additional crosslinks (Friess, 1998), reduced tensile strength (Olde Damink *et al.*, 1995) and gave an enhanced susceptibility to enzymatic degradation (Friess and Schlapp, 2006). Studies have also shown that radiation causes both increased stabilisation and destabilisation of the triple helix (Sionkowska, 2005), which is likely to be the result of both aforementioned chain scission and additional crosslinks. Other DSC analyses have also shown that collagen-based materials display a reduction in denaturation temperature following exposure to  $\gamma$  radiation (Sun and Leung, 2008). Differences between the results presented here and those noted from the literature may be due to the fact that the collagens under investigation originated from different sources.

As well as using temperatures obtained from DSC analyses as an indication of the extent of denaturation, the calorimetric enthalpy can also be considered. This is calculated from area underneath the peak and gives an absolute measurement of the heat energy uptake, which is dependent on the total amount of active protein being analysed. There was a clear similarity between the enthalpies of the as received and the  $\gamma$  irradiated collagens (Figure 2). Statistically, there was no significant difference between the enthalpies of the  $\gamma$  irradiated collagen when compared with the as received collagen ( $p = 0.311$ ), but a significant difference was seen when the as received collagen was compared with both autoclaved ( $p = 0.025$ ) and EtO treated collagen ( $p = 0.006$ ) (Table 1). This finding again suggests that  $\gamma$  irradiation was the least damaging sterilisation treatment. Furthermore, following EtO gas sterilisation, the thermogram peak is much smaller. Smaller peaks, such as this, have been shown to be indicative of protein denaturation (Valencia-Pérez *et al.*, 2008). This is in contrast to the result of no significant difference between the denaturation temperatures of the as received and EtO gas treated collagen, but not wholly unexpected. Changes in the structure of collagen potentially leading to such a change in enthalpy may be due to the susceptibility of some amino acids present in collagen to the alkylation process initiated by EtO (Wiegand *et al.*, 2009), which can reduce the amount of free amino acid groups, resulting in a lower protein binding capacity (Olde Damink *et al.*, 1995).

However, the breadth of the denaturation peak of the  $\gamma$ -irradiated collagen is similar to those of the as received and EtO treated collagens, unlike that for autoclaved collagen which is much narrower. A broad peak, such as that seen in the EtO treated collagen, suggests that the collagen is being denatured gradually. The narrow peak displayed by the autoclaved collagen is likely to be due to extensive denaturation prior to DSC analysis, suggesting there is only a limited amount of structural change that further thermal denaturation can cause following initial thermal treatment by autoclaving.

FTIR can be used to study changes in collagen structure by estimating the triple helix content calculated by ratios of the depths of transmission troughs (Gordon *et al.*, 1974); a method that has been widely used in the literature (Guzzi Plepis, Goissis, and Das-Gupta, 1996; Nagai *et al.*, 2004). Preserving the helical content of collagen is considered a critical requirement for maintaining both the stability and mechanical properties of collagen (Brinkman *et al.*, 2003), which are both likely to play key roles in the *in vivo* behaviour. The peak positions and band intensities associated with amides considered in this study are typical of bovine collagen and also demonstrate a clear correlation with collagen from *C. reniformis* (Heinemann *et al.*, 2007). However, FTIR appears to be less sensitive in identifying differences in collagen from *C. reniformis* caused by various sterilisation processes when compared to some examples in the literature where collagen from other sources was used (Gordon *et al.*, 1974). It is possible that this was due to factors involved in the extraction and production process of compressed collagen fibres from *C. reniformis* or structural differences between this marine collagen and other more widely studied mammalian collagens. However, the technique was able to identify and quantify some differences in the collagen of *C. reniformis* following various sterilisation processes.

The poor FTIR results for EtO sterilisation may be due to the extensive heating and aeration involved in the sterilisation procedure. Both autoclaved and  $\gamma$  irradiated collagens retained their moisture content during the respective sterilisation procedures whereas the EtO processed collagen was completely dry as a consequence of ensuring thorough removal of residual EtO gas. This drying meant that it was difficult to ensure good contact between the sample and the sensor during FTIR analysis resulting in a weak banding pattern. This only partially accounts for the results however, as DSC also highlighted enthalpy changes due to EtO gas sterilisation, which is indicative of collagen denaturation. Nevertheless, differences in the retained moisture content of the collagen, potentially both within and between samples, may have had a limited

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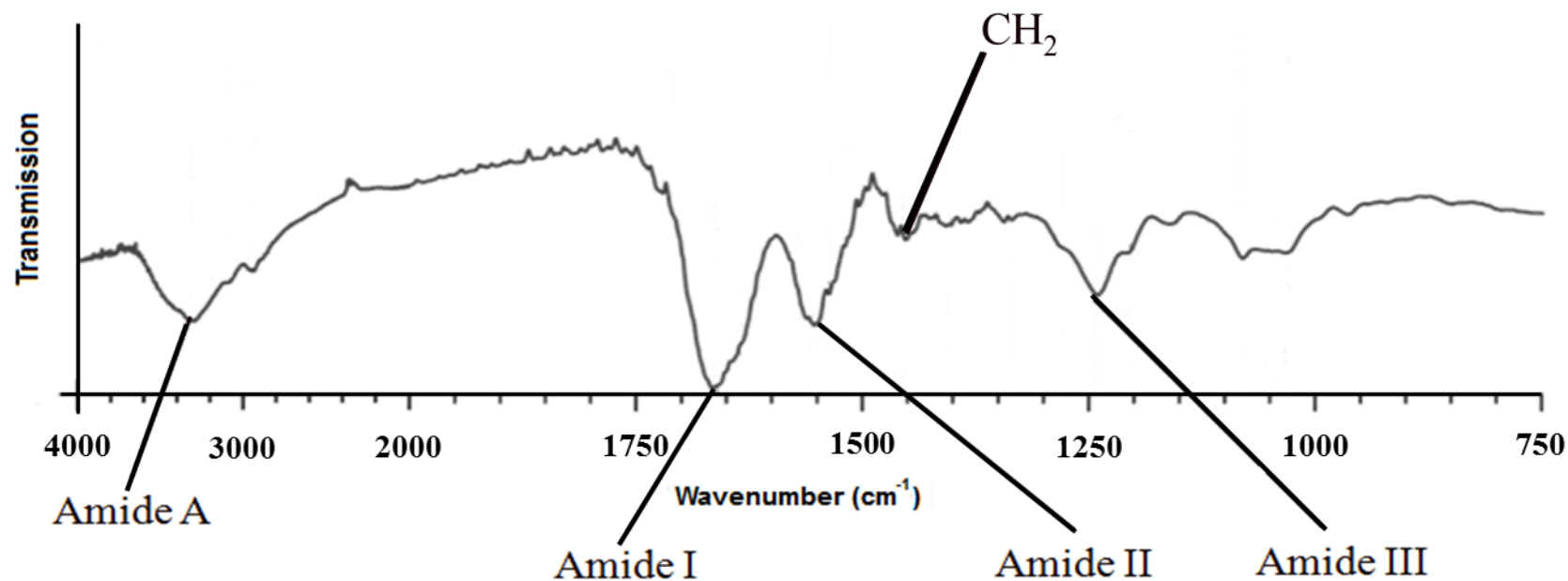
effect on the results obtained from the DSC and FTIR analyses. Any such effect was not considered sufficient to influence the interpretation of the overall trends.

In this study, the FTIR analyses supported those of DSC and found that  $\gamma$  irradiation was the least damaging of the three sterilisation techniques. Both DSC and FTIR showed that autoclaving caused severe denaturation. The results of this study suggest that EtO is not a suitable sterilisation technique for sensitive biomolecules, which is in contradiction with accepted standards within the industry. However, given the relatively low number of samples investigated and the low homogeneity of the collagen, a more comprehensive study using larger sample size should be performed in order to confirm the trends identified in this study and to provide more accurate values for the denaturation temperatures, calorimetric enthalpies and helicity. Also, additional properties of *C. reniformis* collagen, such as its mechanical properties, should be investigated to further understand the effects of sterilisation.

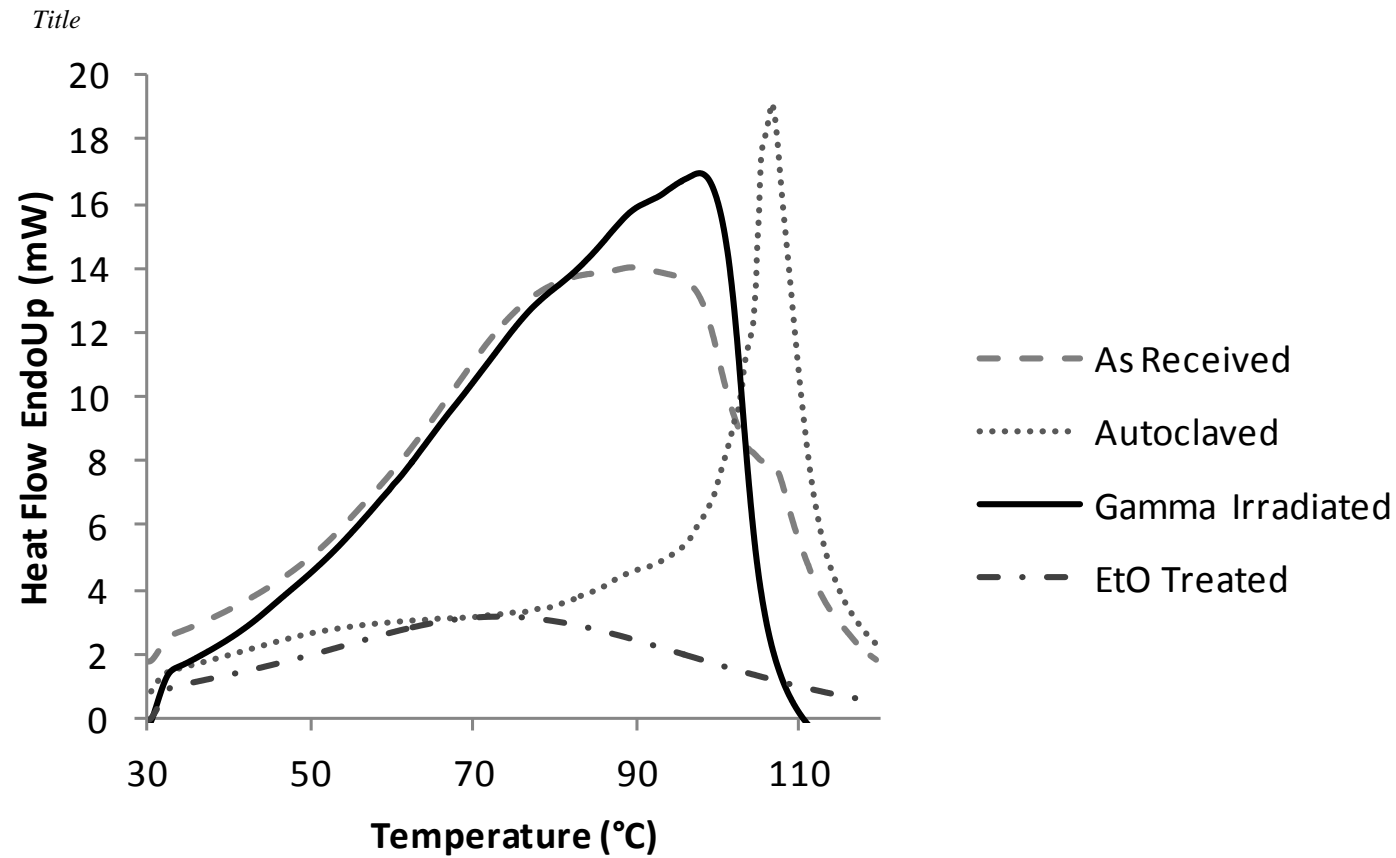
New technologies such as plasmas, vapour-phase hydrogen peroxide, ozone, chlorine dioxide, and high-intensity visible light (Mendes, Brandão, and Silva, 2007) may prove to be less damaging to sensitive biomolecules, but more research is needed before such technologies can be considered as viable alternatives to the techniques investigated herein. Currently, it is necessary to gain as much understanding as possible about the effects any sterilisation technique being considered may have on the biomolecule so that the most appropriate method can be selected.

## CONCLUSION

It is clear that there are many potential effects on the structural properties of marine collagen associated with all common sterilisation techniques. Gaining an understanding of how such sterilisation techniques affect marine collagen is essential before it can be safely and successfully incorporated into biomaterials or medical devices. Based on results from this preliminary study, it is tentatively recommended that  $\gamma$  irradiation is the most suitable sterilisation technique for collagen from *C. reniformis*.



**Figure 1:** Typical banding pattern of collagen from *C. reniformis* with N–H stretching for the amide A (3330 cm<sup>-1</sup>), C=O stretching for the amide I (1634 cm<sup>-1</sup>) and N–H deformations for the amide II (1555 cm<sup>-1</sup>) and III (1235 cm<sup>-1</sup>) bands highlighted, along with the CH<sub>2</sub> deformation band (1450 cm<sup>-1</sup>) which has been shown to be invariant with changes due to denaturation (Heinemann *et al.*, 2007).

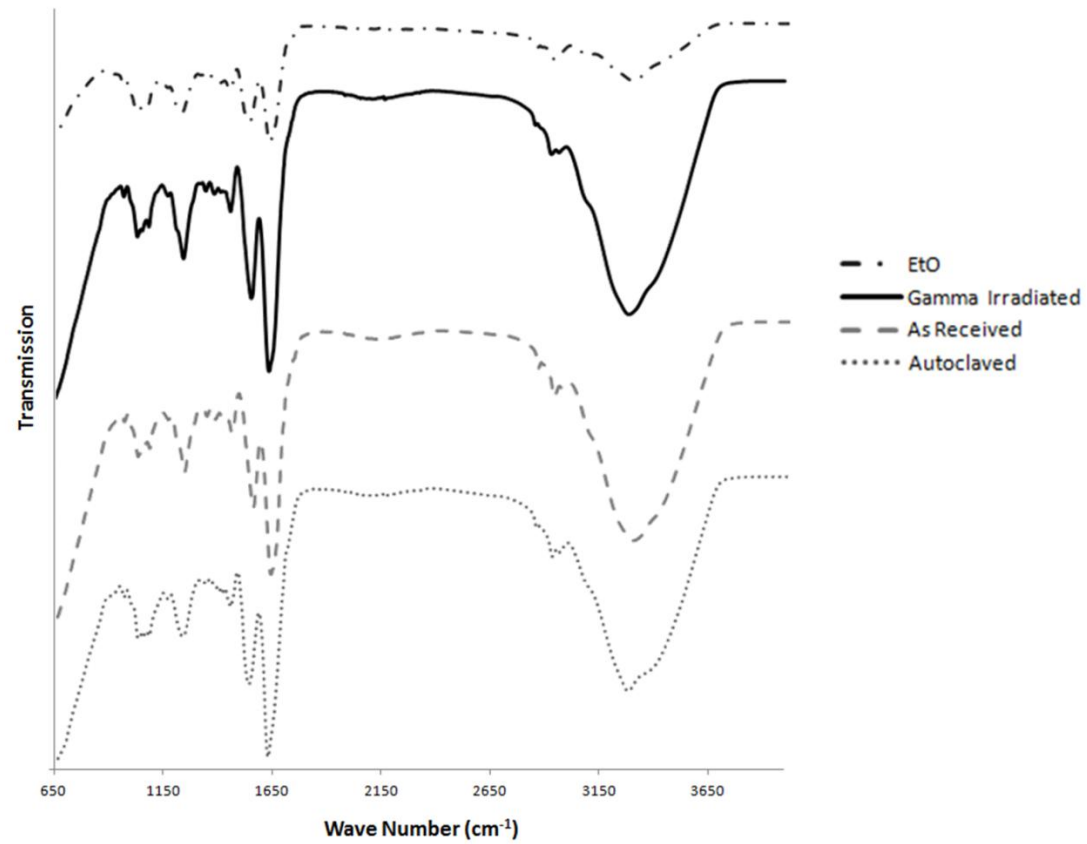


**Figure 2:** Thermal transition curves for as received and treated marine collagens (based on the means of 3 repeats).



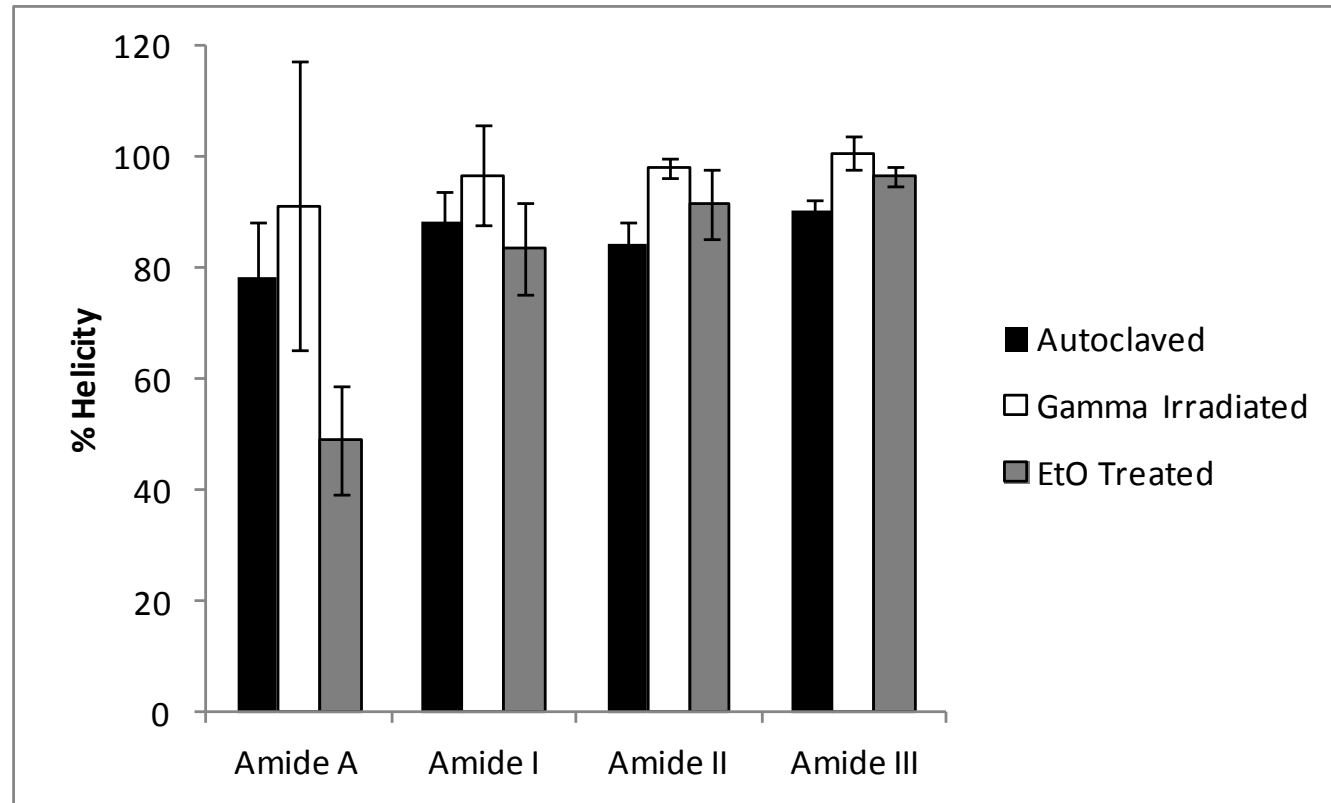
<b>Treatment</b>	<b>Denaturation Temperature (°C)</b>	<b>p-Value</b>	<b>Calorimetric Enthalpy (mJ)</b>	<b>p-Value</b>
As Received	85.9 ± 11.2	N/A	7222.2 ± 2035.7	N/A
Autoclaved	106.1 ± 1.8	0.012	3197.4 ± 1289.7	0.025
EtO	72.9 ± 3.9	0.085	1881.3 ± 254.3	0.006
γ Irradiated	95.7 ± 4.1	0.217	9166.7 ± 1669.9	0.311

**Table 1:** Denaturation temperatures and calorimetric enthalpies (mean ± standard deviation) and respective p-values of as received and treated marine collagens determined from DSC thermal transition curves.

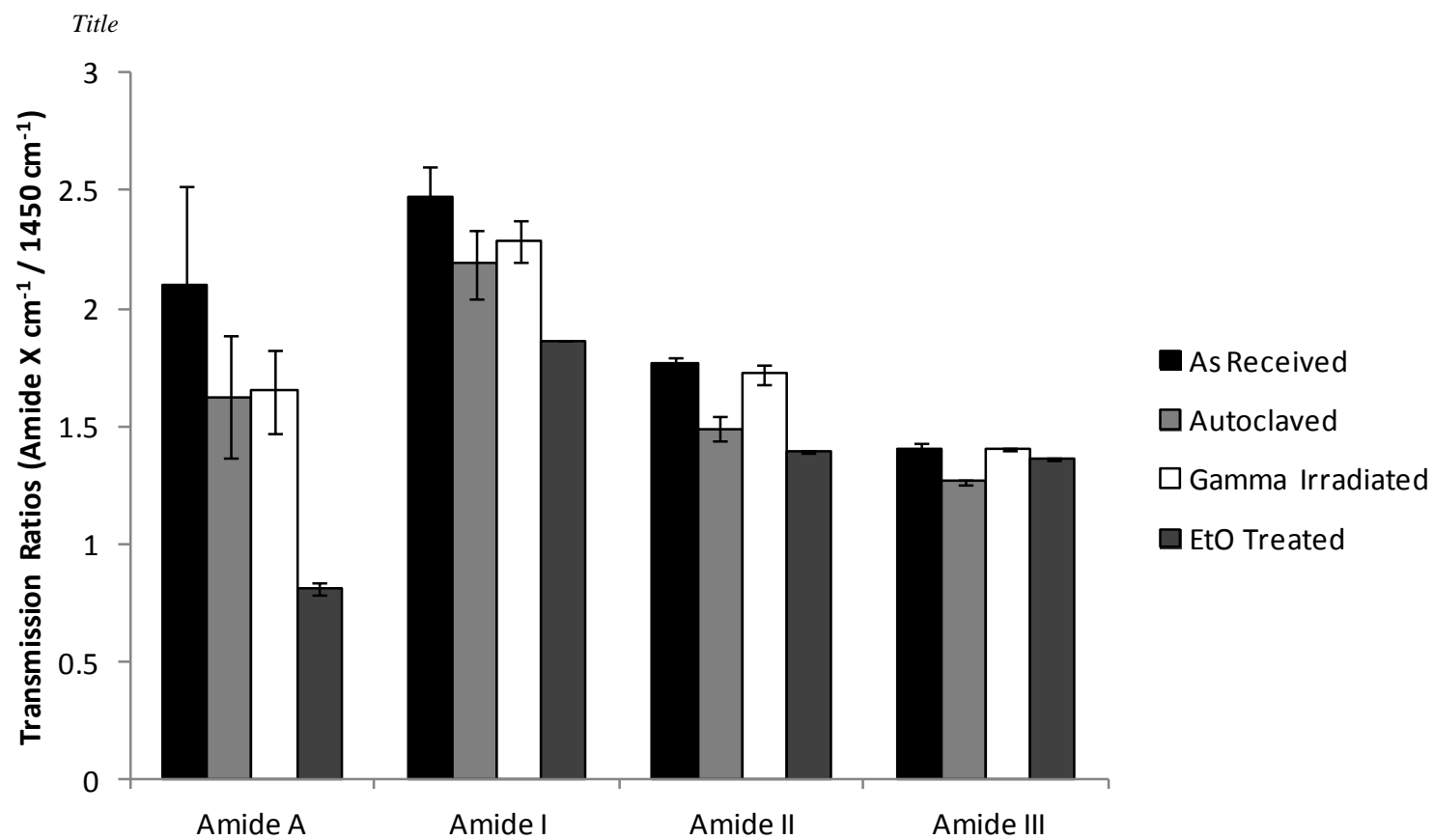


**Figure 3:** FTIR spectra of the as-received and treated marine collagens based on the means of 3 repeats.

Author



**Figure 4:** Helicity (mean  $\pm$  standard deviation) of treated marine collagens as percentages relative to the as received collagen, as indicated by changes in the banding of the respective amide groups.



**Figure 5:** Transmission ratios (mean  $\pm$  standard deviation) of as received and treated marine collagens, as indicated by changes in the banding of the respective amide groups.

<b>Banding</b>	<b>Control Treatment</b>	<b>Sterilisation Treatment</b>	<b>P-Value</b>
<b>Amide A</b>	As Received	Autoclaved	0.226
		EtO Treated*	0.002
		$\gamma$ Irradiated	0.680
<b>Amide I</b>	As Received	Autoclaved	0.091
		EtO Treated*	0.001
		$\gamma$ Irradiated	0.815
<b>Amide II</b>	As Received	Autoclaved*	< 0.001
		EtO Treated*	< 0.001
		$\gamma$ Irradiated	0.693
<b>Amide III</b>	As Received	Autoclaved*	< 0.001
		EtO Treated	0.064
		$\gamma$ Irradiated	0.873

**Table 2:** Results of statistical analyses comparing all sterilisation treatment methods to as received collagen for each of the four amide groups (\* denotes a significant difference).

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